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Somatic Cell Mutations at the Glycophorin A Locus in Erythrocytes of Radiation Workers from the Sellafield Nuclear Facility

E. Janet Tawn,[a,1] Caroline A. Whitehouse,[a] C. Paul Daniel,[a] Robert E. Tarone,[b] Andrea M. Bothwell[a] and Ann Fisher[a]

[a] *Genetics Unit, Westlakes Research Institute, Cumbria, United Kingdom; and* [b] *Division of Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland*

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The glycophorin A (GPA) somatic mutation assay for N0 and NN mutant erythrocytes was performed on 245 current and 48 retired workers who had been occupationally exposed to radiation at the British Nuclear Fuels plc facility at Sellafield. A positive association with increasing age was found for current workers for both N0 and NN frequencies of $0.14 \pm 0.05 \times 10^{-6}$ ($P = 0.012$) and $0.25 \pm 0.07 \times 10^{-6}$ ($P = 0.0003$) per year, respectively. No association with age was found for the retired workers. In a comparison of ever-smokers with never-smokers, no difference was observed for N0 frequencies for current workers, but a significantly higher frequency was found for ever-smokers in the retired group ($P = 0.001$). NN mutant frequencies were slightly higher in ever-smokers than in never-smokers for both current and retired workers, but in neither case was the increase significant. In age-adjusted analyses for N0 mutant frequencies, a slight positive radiation dose response was found for current workers ($1.6 \pm 3.8 \times 10^{-6}$ per Sv), for retired workers ($2.9 \pm 2.5 \times 10^{-6}$ per Sv), and in the combined analysis ($2.6 \pm 2.2 \times 10^{-6}$ per Sv), but in no case did this reach significance. Similar analyses for NN mutant frequencies revealed a positive dose response for current workers ($4.7 \pm 4.6 \times 10^{-6}$ per Sv) and a negative response for retired workers ($-2.4 \pm 3.6 \times 10^{-6}$ per Sv) that was maintained in the combined analysis ($-1.4 \pm 2.8 \times 10^{-6}$ per Sv), but none of these slopes was significantly different from zero. The results suggest that the GPA mutation assay is insufficiently sensitive to be used as a biological marker of low-dose chronic exposure and provide further evidence that, in contrast to high acute radiation exposure, protracted exposure is much less effective at inducing somatic mutations *in vivo*.

INTRODUCTION

Although the long-term health effects associated with radiation exposure are well documented (*1, 2*), the risks are difficult to quantify for very low doses and low dose rates because an observable effect cannot be measured easily even in large exposed populations. Thus the epidemiological data which form the basis of quantitative assessments of health risk come from high-dose studies with a dose and dose-rate effectiveness factor (DDREF) being applied to derive risks for low doses (*1, 2*). In recent years, progress in the understanding of both the etiology of cancer and the molecular consequences of radiation exposure has suggested that induction of somatic mutations, i.e. gene mutations and chromosome rearrangements, is a key event common to both processes (*2–4*). The use of these types of genetic change as biomarkers of exposure provides the opportunity to make dose–response comparisons of different exposure conditions that are of direct relevance to risk estimation.

The glycophorin A (GPA) somatic mutation assay uses flow cytometry to measure the frequency of mutant red blood cells from individuals who are heterozygous for the two GPA alleles, M and N. Two sorts of mutant cells are detectable, N0, which result from deletion or inactivation of the M allele, and NN, which require both the loss of the M allele and the duplication of N (*5*). Increased frequencies of N0 cells have been observed in several studies of populations exposed to acute high-dose, high-dose-rate radiation including the survivors of the Japanese atomic bombs (*6–10*), the Chernobyl cleanup workers (*11–13*), individuals accidentally exposed to a ^{137}Cs source in Brazil (*14*), and patients receiving therapeutic radioiodine (*15*). Studies of chronic or low-dose exposures have been more difficult to interpret. An increase in N0 frequencies was found in immigrants to the U.S. from the Chernobyl area who had measurable levels of internal radiocesium (*16*), but studies of liquidators have failed to find significant increases at low doses (*11, 13, 17–21*), and the minimum dose at which an increase was detectable in the Japanese atomic bomb survivors was 0.24 Sv (*9, 10*). In contrast, a recent study of Korean nuclear power plant workers and hospital workers exposed to low-dose radiation revealed a significant increase in N0 frequency associated with cumulative radiation dose (*22*). Analysis of a group of men exposed occupationally within permitted limits at the British Nuclear Fuels plc (BNFL) Sellafield nuclear facility, Cumbria, UK,

[1] Author to whom correspondence should be addressed. Genetics Unit, Westlakes Research Institute, Moor Row, Cumbria, CA24 3JY, UK; e-mail: Jan.Tawn@westlakes.ac.uk.

found a weak positive association with dose, but the increase was not significant (*23*). Since then a considerable amount of data has been accumulated on GPA mutant frequencies for both current and retired Sellafield workers, and the data on this larger population form the basis of the present study.

MATERIALS AND METHODS

Participants

This work was reviewed and approved by the West Cumbria Local Research Ethics Committee in accordance with UK regulations. The study involved two groups of workers who had a history of occupational radiation exposure, current employees and retired workers. Estimates of cumulative external dose for all the workers were based on film badge records. Some individuals had additional localized doses due to internally deposited radionuclides, but these were small when compared to recorded whole-body doses. Individuals known to have received radiotherapy were excluded from the study.

In the first phase of the study, conducted between 1993 and 1996, blood samples were obtained after informed consent from 297 current male radiation workers who were heterozygous for the M and N alleles. These volunteers were not selected by dose, and their date of birth and smoking history were supplied by the Department of Occupational Health at Sellafield. A further series of male radiation workers who had previously worked at Sellafield were recruited between 1997 and 1998 as part of a cytogenetic study of retired workers (*24*). Samples were obtained from 45 MN heterozygotes with cumulative lifetime doses of >500 mSv and 32 MN heterozygotes with lifetime doses of <50 mSv. Data on age and smoking habits for this group were collected using a questionnaire.

GPA Assay

Samples were allocated a study number and analyzed blind. GPA expression mutants were measured, usually within a week of collection, using a method described previously (*25*) that was adapted from Langlois *et al.* (*26*). Briefly, the GPA alleles M and N were typed using a standard hemagglutination assay, and only samples from MN heterozygotes were fixed for analysis. Fixed red blood cells were labeled with a pair of monoclonal antibodies specific for the M and N antigens, respectively (anti-M, biotinylated 6A7; anti-N, fluoroscein conjugated Bric 157, IBGRL, Elstree, UK). Binding of the anti-M antibody was revealed indirectly using streptavidin-phycoerythrin (Becton Dickinson). Mutant cells were analyzed in a Becton Dickinson FACstar Plus flow cytometer using forward and orthogonal light scatter to define the red blood cell population and fluorescence at 575 nm (orange) and 530 nm (green) to detect binding of the anti-M and anti-N antibodies, respectively. Normal heterozygous cells bind both antibodies and were detected as emitting both green and orange fluorescence. Mutant cells of the N0 gene loss type were defined as normal for green fluorescence but less than 1% of the peak channel value for orange fluorescence. Loss and duplication NN cells were also negative for orange fluorescence but were distinguished from N0 cells by an approximate doubling of green intensity.

Normally 4 million red blood cells were analyzed for each sample, although a small number had up to 11 million cells analyzed; in a few cases the quality of the blood sample restricted the analysis to between 1.2 and 4 million cells. Each scatter plot of N fluorescence compared to M fluorescence was examined before the results were calculated. Some plots were rejected because a ''tail'' of cells with low-intensity M fluorescence encroached on the analysis region. This phenomenon has been described previously (*26*), although the number of samples affected has not routinely been reported in other studies. This resulted in the rejection of 42 samples from the current worker group and 20 samples from the retired worker population, 8 from the <50-mSv group, and 12 from the high-dose >500-mSv group. Listmode files containing data on the fluorescence intensity of individual cells were transferred to a program supplied by T. Hoy (Department of Haematology, University Hospital of Wales) for calculation of the number of N0 and NN cells according to the specification described by Langlois *et al.* (*26*). Thus GPA mutant frequencies were obtained successfully for 255 current workers and 57 retired workers.

Statistical Methods

Statistical analyses were performed using the Generalized Linear Interactive Modelling System (GLIM, Royal Statistical Society, c/o NAG, 256 Banbury Road, Oxford, UK). Analyses of GPA mutant frequencies were performed using standard multiple linear regression methods (i.e. the normal error structure). Analyses using categorical and continuous variables for dose and age gave comparable results. Only the results based on analyses of continuous variables are presented (i.e. the slope $\pm$ standard error for cumulative dose and for age). Analyses were performed separately for current workers and for retired workers, and a combined analysis for all workers (i.e. including an indicator variable for work status) was performed to obtain summary slope estimates. Two-sided P values are reported.

RESULTS

A small proportion (10/255, 3.9%) of samples from the current worker group were found to have high numbers of NN cells, ranging from 50 to 455 per million cells, some of which strayed into the adjacent flow cytometric analysis region and interfered with the accurate measurement of the N0 frequency. A slightly higher proportion (9/57, 16%) of samples from retired workers were NN outliers. Seven were from the high-dose group and two from the low-dose group. These NN outliers were excluded, leaving 245 current workers and 48 retired workers for the following analysis. Data on N0 and NN mutant frequencies for the two groups of workers, current and retired, together with that for the total study group, are presented in Table 1 in three dose categories, <50 mSv, 50–500 mSv and >500 mSv. Analysis for the effect of age revealed a positive association with increasing age for current workers for both N0 and NN variant frequencies with slopes per year of age of $0.14 \pm 0.05 \times 10^{-6}$ ($P = 0.012$) and $0.25 \pm 0.07 \times 10^{-6}$ ($P = 0.0003$), respectively. Adjustment for cumulative dose changed these slope estimates only slightly. No association with age was found for the retired workers. Current smokers and ex-smokers had similar variant frequencies. In a comparison of ever-smokers with never-smokers, no effect of smoking was observed for N0 frequencies for current workers, but a significantly higher frequency was found for ever-smokers in the retired group ($P = 0.001$). NN mutant frequencies were slightly higher in ever-smokers than in never-smokers for both current and retired workers, with a greater effect being observed in the retired workers, but in neither case did the increase approach statistical significance.

In age-adjusted analyses for N0 mutant frequencies a slight positive radiation dose response was found for current workers ($1.6 \pm 3.8 \times 10^{-6}$ per Sv), for retired workers ($2.9 \pm 2.5 \times 10^{-6}$ per Sv), and in the combined analysis

TABLE 1
GPA N0 and NN Mutant Frequencies

Dose group	Number of individuals	Number of ever-smokers	Mean dose (mSv) (range)	Mean age (years) (range)	Mean N0 mutant frequency $\times 10^{-6}$ (range)	Mean NN mutant frequency $\times 10^{-6}$ (range)
<50 mSv						
Current workers	129	53	16 (0–50)	39 (18–60)	8.90 (0.50–38.50)	8.91 (0–46.75)
Retired workers	22	15	16 (0–48)	70 (57–84)	10.36 (2.25–20.83)	16.08 (2.25–44.00)
Combined	151	68	16 (0–50)	44 (18–84)	9.12 (0.50–38.50)	9.96 (0–46.75)
50–500 mSv						
Current workers	110	60	161 (53–489)	44 (28–60)	9.64 (0.25–58.50)	9.40 (0–47.75)
>500 mSv						
Current workers	6	5	665 (504–935)	53 (44–60)	13.16 (6.75–22.00)	18.42 (3.75–46.92)
Retired workers	26	17	751 (500–1656)	71 (57–89)	11.62 (0–37.91)	10.69 (0–33.25)
Combined	32	22	735 (500–1656)	68 (44–89)	11.91 (0–37.91)	12.14 (0–46.92)

($2.6 \pm 2.2 \times 10^{-6}$ per Sv), but in no case did this positive response reach significance. Similar analyses for NN mutant frequencies revealed a positive dose response for current workers ($4.7 \pm 4.6 \times 10^{-6}$ per Sv) and a negative response for retired workers ($-2.4 \pm 3.6 \times 10^{-6}$ per Sv) that was maintained in the combined analysis ($-1.4 \pm 2.8 \times 10^{-6}$ per Sv), but none of these slopes were significantly different from zero.

DISCUSSION

This study has investigated the relationship between GPA mutant frequency and radiation dose in an extended population of radiation workers with occupational dose data based on physical dosimetry. Information on smoking and age has also been considered in the analysis. A distinctive feature of the study population is that reliable individual dose records exist for relatively low doses acquired over a prolonged period. Unlike studies of accidental or acute exposures, therefore, the investigation is directly relevant to the derivation of risks of potential health effects of the more commonly encountered situation of low-dose chronic irradiation. A preliminary analysis of some of the data in the present study has been reported previously (*27*), but this was undertaken before individual dosimetry and personal details could be validated. In addition, a number of men with doses >500 mSv whose samples were taken during the development stage of the assay and analyzed over a year later were included in the earlier analysis, and we now have doubts about the validity of the results obtained on these samples. Thus the analysis presented here supersedes the earlier report.

A number of studies have examined the effect of age on mutant frequencies. A significant positive association between age and combined N0 and M0 frequency was found in an analysis of 379 healthy Japanese MN heterozygotes, with the increase being about 3% per year (*28*), and this was attributed to a simple accumulation of mutations in stem cells over time. MM mutant frequencies also increased but at a lower rate. A slight but significant age-related increase in M0 and N0 frequencies was also found for a group of Japanese atomic bomb survivors aged 40 to 90 years (*9, 10*), with the increase being 0.7% per year. However, studies of Chernobyl cleanup workers have produced conflicting results. Analysis of GPA mutations in samples from Estonian and Latvian liquidators demonstrated a significant age effect for both N0 and NN mutant frequencies (*18*). In contrast, an earlier study of Chernobyl cleanup workers from Ukraine and Russia found no association between age and N0 frequencies and only a marginal age effect for NN frequencies (*11*), nor was an association found in a later study from Russia (*19*). No significant effect of age was found for either N0 or NN mutant frequencies in a previous study of 36 workers exposed to occupational radiation at Sellafield (*23*). In the present study we have demonstrated a significant increase for N0 mutant frequencies with age for the current worker group, which had an age range of 18 to 60 years, but not for the older retired group, whose age range was 57 to 89 years. While this might suggest that mutant frequencies reach a plateau around age 60, this would not be consistent with data on another well-established measure of somatic mutation, namely chromosome translocations (*29–31*).

Few studies have examined the effect of smoking, with the only positive association being reported for the Japanese atomic bomb survivors (*9, 10*). No effect of smoking was seen in two studies of Chernobyl cleanup workers (*11, 19*), and although there was a suggestion of an effect in the earlier study of Sellafield workers, this was not significant (*23*). In the present study, a significantly increased N0 variant frequency associated with smoking was found for the retired workers but not for the current workers. Whether this reflects differences in smoking history is unclear, particularly since difficulties in quantifying smoking habits always make the interpretation of results problematic. Smoking did not appear to influence NN mutant frequencies in the present study.

Samples from two groups of workers with different patterns of exposure in relation to time of blood sampling were analyzed for GPA mutations in this study, 245 who were

in current employment and 48 retired employees. The results from the two groups were initially analyzed separately. However, dose-related increases in GPA N0 mutant frequencies have been demonstrated in the Japanese atomic bomb survivors some 40 years after exposure, thus illustrating that exposure to ionizing radiation induces long-lived mutations at the GPA locus in progenitor hemopoietic cells (*6–10*). Furthermore, linear regressions of N0 mutant frequency as a function of dose for the atomic bomb survivors yield slopes similar to those derived from studies of Chernobyl cleanup workers receiving acute high-dose exposures, conducted within several years of the accident (*11, 13*). The concordance of the acute dose responses for A-bomb survivors and Chernobyl cleanup workers led Jensen *et al.* (*11*) to conclude that GPA mutations in the bone marrow are stable and continue to produce variant progeny in the peripheral blood for many years after exposure. This conclusion is further supported by repeated sampling of exposed individuals from Chernobyl over a 7-year period (*11*) and of A-bomb survivors over a 5-year period (*10*). No decrease in mutant frequency over time was detected for either exposure group. In a study of outliers with elevated frequencies identified from an analysis of healthy individuals with no known significant exposures to physical or chemical mutagens, the high frequencies were maintained in subsequent sequential sampling (*32*). Thus temporal differences in patterns of exposure in relation to blood sampling do not appear to influence the frequencies of mutant cells in peripheral blood erythrocytes, and the persistence of mutant frequencies has enabled the GPA mutation assay to be used as a biological dosimeter for the assessment of doses in cases where accurate physical measurements were not available (*14, 18, 33*). In view of this, no attempt was made in this study to examine the exposure profiles, and although the study subjects were initially examined in two groups, i.e. current and retired workers, the groups were then combined. Although a small positive dose response for the total study population of $2.6 \pm 2.2 \times 10^{-6}$ per Sv was observed, this did not reach significance.

Whereas reports of high-dose acute exposures clearly demonstrate an increase in GPA N0 mutant frequency associated with radiation dose, studies of low doses or chronic exposures have been more difficult to interpret. An analysis of 1226 survivors from Hiroshima and Nagasaki revealed a linear regression dose response of 19.6×10^{-6} per Sv (*9, 10*), and among Chernobyl liquidators who had suffered from acute radiation sickness, a value of 22×10^{-6} per Gy was reported (*11*). The latter study also examined the dose response for those who had received <1 Gy of radiation, and a value 4.1×10^{-6} per Gy was obtained, although the regression was not significantly different from zero. This value was also consistent with the dose regression of GPA N0 mutant frequency observed in a group of Estonian Chernobyl cleanup workers with doses ranging up to around 300 mSv, but this was of only marginal significance, and no association with dose was seen for a similar group of Latvian cleanup workers studied at the same time (*18*). A study of 79 Ukrainian Chernobyl cleanup workers exposed to low doses of radiation found no increase associated with exposure (*19*). However, the same study reported a biological assessment of the mean dose to the group, based on chromosome aberration analysis, of 90 mGy, suggesting that the proposed mean physical dose of 250 mSv was an overestimate. This lack of response for Chernobyl cleanup workers has recently been confirmed in an extensive study of 370 Russians who served as liquidators after the accident (*21*). However, a significant positive dose response of 6.3×10^{-6} per Gy has been reported for a smaller study of 46 radiation workers who had been exposed to prolonged irradiation (*20*). The group included both Chernobyl cleanup workers and workers with doses of several grays accumulated over many years. Restricting the analysis to the Chernobyl cleanup workers who had received doses of <250 mGy revealed no significant difference in mutant frequency values in comparison with unirradiated controls. In a further analysis, an acute dose response of 32×10^{-6} per Gy was found for a limited data set of individuals with accidental exposures in comparison with a dose response of 9.6×10^{-6} per Gy for protracted exposure, and it was suggested that while the technique is suitable for determining the extent of historical protracted exposures, it will be applicable only for doses of 1 Gy or above (*13*). In contrast, a recent study of 144 nuclear power plant workers in Korea, with cumulative doses ranging up to 120 mGy, found a significant positive dose response for N0 mutant frequency of 17.5×10^{-6} per Gy (*22*), which is somewhat higher than the general trend found in studies of populations exposed to low-dose chronic exposure. Furthermore, a parallel study of 32 hospital workers resulted in a dose response approximately 10 times higher, i.e. 188.1×10^{-6} per Gy, although the authors acknowledge that this remarkably high value is driven by one individual. In a previous investigation of 36 Sellafield workers with doses in the range 2–990 mSv (*23*), a dose–response value for N0 mutant frequency of 12.2×10^{-6} per Sv was derived, but the association was weak and not significant. In the present larger study of 293 workers with chronic low-dose occupational exposure, the positive response of $2.6 \pm 2.2 \times 10^{-6}$ per Sv is not significant and is thus consistent with the results from the majority of population studies of low-dose radiation exposure.

The N0 mutant cells detected in the GPA mutation assay arise as a result of loss-of-function mutations through base substitution or gene deletion. Such mutational mechanisms also give rise to the changes in tumor suppressor genes that are associated with oncogenesis. Since ionizing radiation is most effective at inducing large DNA deletions, it has been proposed that loss-of-function events will be the predominant process in radiation-induced carcinogenesis (*1*). Thus the dose response for radiation-induced GPA N0 mutant cells will reflect the probability of the induction of tumor suppressor gene mutations. Furthermore, it has been sug-

gested that the frequency of N0 cells might itself be a useful end point for assessing cancer risk, with support for this coming from the analysis of mutant frequencies in A-bomb survivors, where a significantly higher dose response was found for persons diagnosed with cancer than for cancer-free individuals (*10*). Loss-of-function tumor suppressor gene mutations associated with chromosome deletions are the predominant genetic changes associated with solid tumors (*34*). The present study examined N0 frequencies in a total of 293 individuals exposed occupationally to radiation with cumulative doses ranging from 0 to 1656 mSv. The suggestion of a low positive dose response of $2.6 \pm 2.2 \times 10^{-6}$ per Sv, which did not even reach significance, is in marked contrast to those of the order of $20–30 \times 10^{-6}$ per Gy derived from studies of populations exposed to high acute doses of radiation. This suggests that chronic low-dose exposure risks derived for solid tumors that apply a dose and dose-rate effectiveness factor (DDREF) of 2 to data obtained from the study of populations with high-dose acute exposures are conservative.

Previous studies of Sellafield radiation workers have employed somatic mutation analysis and cytogenetic techniques to measure mutation frequencies. Cole *et al.* (*35*) examined mutation frequencies at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus in 18 Sellafield workers with cumulative exposures >450 mSv and compared them to 18 matched workers with exposures <50 mSv. No increase associated with exposure was found. Indeed, an inverse association with dose of borderline statistical significance was reported, which the authors interpreted as representing a statistical artifact obtained by fitting too many parameters to too small a data set. More extensive investigations have involved chromosome aberration analysis. Parallel investigations by Tucker *et al.* (*23*) and Tawn *et al.* (*36*) employed fluorescence *in situ* hybridization (FISH) and G-banded chromosome analysis, respectively, to measure the frequency of stable chromosome aberrations in the same population of workers exposed to external radiation. Both studies found similar significant dose-dependent increases in the frequency of stable chromosome aberrations, principally translocations, which were of the order of six to eight times lower than the dose response observed in a study of the Japanese atomic bomb survivors. Our present findings support the view (*21*) that the GPA mutation assay, which initially showed considerable promise for monitoring populations exposed to radiation, is not as sensitive as the analysis of chromosome translocations, and therefore cytogenetic analysis remains the preferred technique for detecting exposures to low doses of radiation. Nevertheless, the study provides further evidence that the induction of somatic genetic damage by low-dose chronic exposure is less per unit dose than for acute high-dose irradiation. As more attention is focused on the mechanistic basis of risk estimation, such findings should be evaluated when extrapolating risk factors derived from studies of highly exposed populations to the occupational exposure situation.

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